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(54) Rapid detection of nucleic acid sequences in a sample by labeling the sample.

(57) A method for detecting one or more microorganisms or polynucleotide sequences from eukaroytic sources in a nucleic acid-containing test sample comprising

(a) preparing a test sample comprising labeling the nucleic acids in the test sample,

(b) preparing one or more process by immobilizing a single-stranded nucleic acid of one or more known microorganisms or sequences from eukaroytic sources,

(c) contacting, under hybridization conditions, the labeled single-stranded nucleic acid to form hybridized labeled nucleic acids, and

(d) assaying for the hybridized nucleic acids by detecting the label. The method can be used to detect genetic disorders, e.g., sickle-cell anemia.

EP 0 235 726 A2

RAPID DETECTION OF NUCLEIC ACID SEQUENCES IN A SAMPLE BY LABELING THE SAMPLE

BACKGROUND OF THE INVENTION

Field of the Invention

The present application relates to the detection and identification of microorganisms and the detection and identification of particular prokaryotic or eukaryotic DNA sources in a nucleic acid containing test sample.

Still further, the present invention relates to a method for the lysis of whole cells.

Background Information

A. The Detection of Microorganisms

The identification of species of microorganisms in a sample containing a mixture of microorganisms, by immobilizing the DNA from the sample and subjecting it to hybridization with a labelled specimen of species -specific DNA from a known microorganism and observing whether hybridization occurs between the immobilized DNA and the labelled specimen, has been disclosed in PCT patent application No. PCT/US83/01029.

The most efficient and sensitive method of detection of nucleic acids such as DNA after hybridization requires radioactively labeled DNA. The use of autoradiography and enzymes prolongs the assay time and requires experienced technicians.

U.S.P. 4,358,535 to Falkow et al describe infectious disease diagnosis using labeled nucleotide probes complementary to nucleic acid coding for a characteristic pathogen product.

B. The Detection of Specific Eukaryotic Sequences

The identification of specific sequence alteration in an eukaryotic nucleic acid sample by immobilizing the DNA from the sample and subjecting it to hybridization with a labeled oligonucleotide and observing whether hybridization occurs between the immobilized DNA and the labeled probe, has been described in EP -patent application No. 86 117 978 filed December 23, 1986, now pending.

It is known that the expression of a specific gene determines the physical condition of a human being. For example, a change in the beta-globin gene coding sequence from GAG to GTG at the sixth amino acid position produces sickle-beta-globin and a homozygote can have a disease known as sickle cell anemia. Similarly deletion of particular sequences from alpha-globin or beta-globin genes can cause thalassemias. A recent survey, The New Genetics and Clinical Practice, D.J. Weatherall, The Nuffield Provincial Hospitals Trust, (1982), chapter 2 describes the frequency and clinical spectrum of genetic diseases.

Problems associated with genetic defects can be diagnosed by nucleic acid sequence information. The easiest way to detect such sequence information is to use the method of hybridization with a specific probe of a known sequence.

U.S.P. 4,395,486 to Wilson et al describe a method for the direct analysis of sickle cell anemia using a restriction endonuclease assay.

Edward M. Rubin and Yuet Wai Kan, "A Simple Sensitive Prenatal Test for Hydrops Fetalis Caused By α-Thalassaemia", The Lancet, January 12, 1985, pp. 75-77 describes a dot blot analysis to differentiate between the genotypes of homozygous alpha-thalassemia and those of the haemoglobin-H disease and alpha-thalassemia trait.

The most efficient and sensitive method of detection of nucleic acids, such as DNA, after hybridization requires radioactively labelled DNA. The use of autoradiography and enzymes prolongs the assay time and requires experienced technicians.

Recently, a non-radioactive method of labelling DNA was described by Ward et al, European Patent Application 63,879. Ward et al, use the method of nick translation to introduce biotinylated U (uracil) residues into DNA, replacing T (thymine). The biotin residue is then assayed with antibiotin antibody or an avidin-containing system. The detection in this case is quicker than autoradiography, but the nick translation

method requires highly skilled personnel. Moreover, biotinylation using biotinylated UTP (uridine triphosphate) works only for thymine-containing polynucleotides. The use of other nucleoside triphosphates is very difficult because the chemical derivatization of A (adenine) or G (guanine) or C (cytosine) (containing $-NH_2$) with biotin requires the skills of trained organic chemists.

C. Cell Lysis

The present invention also provides a method for the efficient lysis of whole cells such that their DNA is released and made available for photochemical labeling. While eukaryotic cells derived from multicellular animals are easily lysed under relatively mild conditions, single cell eukaryotes and prokaryotes, especially Gram positive prokaryotes, are more difficult to lyse due to the complicated chemical nature and extent of cross-linking of their cell walls. Methods do exist for efficiently lysing these refractory organisms, either by chemical-enzymatic or physical means, but these methods are often complicated, time-consuming and inappropriate for preserving the integrity of DNA.

SUMMARY OF THE INVENTION

It is accordingly an object of the present invention to provide a method for detection of microorganisms in a nucleic acid-containing test sample.

It is another object of the invention to provide a method for a simultaneous assay for the presence of more than one nucleic acid sequence.

Another object is to provide a method to identify particular prokaryotic or eukaryotic DNA sequences and a method for distinguishing alleles of individual genes.

Another object of the invention is to provide a simple photochemical method of labeling the unknown test sample.

A further object of the invention is to label the probes with different kinds of labels so that when the probes are hybridized with an immobilized, unknown, unlabelled test sample, the type of label remaining bound after hybridization and washing, will determine the type of nucleic acid sequence present in the unknown sample.

A still further object of the invention is to use whole chromosomal nucleic acid as the probe and/or as the test sample.

Also the invention relates to the use of oligonucleotides as immobilized probes.

These and other objects and advantages are realized in accordance with the present invention for a method of detecting nucleic acid sequences in a nucleic acid-containing test sample.

The method involves the following:

(a) preparing a test sample comprising labeling the nucleic acids of the organisms or cells or cell debris in the test sample,

(b) preparing one or more probes by immobilizing a single-stranded DNA or an oligonucleotide of one or more known microorganisms or eukaryotes, or sequences representing particular genes or their alleles,

(c) contacting, under hybridization conditions, the labeled single-stranded sample nucleic acid and the immobilized single-stranded (probe) nucleic acid or the immobilized oligonucleotide to form hybridized labeled nucleic acids and

(d) assaying for the hybridized nucleic acids by detecting the label.

In the above method, steps (a) and (b) can be reversed.

The method further comprises denaturing the labeled nucleic acids from step (a) to form labeled denatured nucleic acids.

According to the invention, a labeled nucleic acid test sample is contacted simultaneously with several different types of DNA probes for hybridization. The nucleic acid test sample is labeled and hybridized with several unlabeled immobilized probes. The positions of the probes are fixed, and the labeled probe detected after hybridization will indicate that the test sample carries a nucleic acid sequence complementary to the corresponding probe.

Nucleic acid probes for several microbiological systems or for different alleles of one or more genes can be immobilized separately on a solid support, for example, nitrocellulose paper. The test sample nucleic acids are labeled and remain in solution. The solid material containing the immobilized probe is brought in contact with the labeled test nucleic acid solution under hybridization conditions. The solid

material is washed free of unhybridized nucleic acid and the label is assayed. The presence of the label with one or more of the probes indicates that the test sample contains nucleic acids substantially complementary to those probes and hence originate, for example, from an infection by particular microbiological systems.

Labeling can be accomplished in a whole living cell or a cell lysate, and can be non-isotopic. The nucleic acid can be used for hybridization without further purification.

The present invention also concerns specific lysis conditions to release nucleic acids from both gram positive and gram negative bacteria.

The present invention further concerns a kit for detecting microorganisms or eukaryotes in a test sample comprising

(a) a support solid containing single-stranded DNA of one or more known microorganisms or eukaryotes immobilized thereon, e.g., a strip containing dots or spots of known microorganisms or eukaryotes,

(b) a reagent for labeling the nucleic acid of the test sample,

(c) a reagent for releasing and denaturing DNA in the test sample, and

(d) hybridization reagents.

For chemiluminescence detection of the hybridized nucleic acid, the kit would further comprise a reagent for chemiluminescent detection.

In the above described kit, the reagent for labeling is given hereinbelow in a discussion on labels.

Reagents for releasing and denaturing DNA include sodium hydroxide and lysing agents such as detergents and lysozymes.

Typical hybridization reagents includes a micture of sodium chloride, sodium citrate, SDS (sodium dodecyl sulfate), bovine serum albumin, nonfat milk or dextran sulfate and optionally formamide.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is an autoradiograph of results of immobilization of an oligonucleotide sequence specific for hemoglobin mutation.

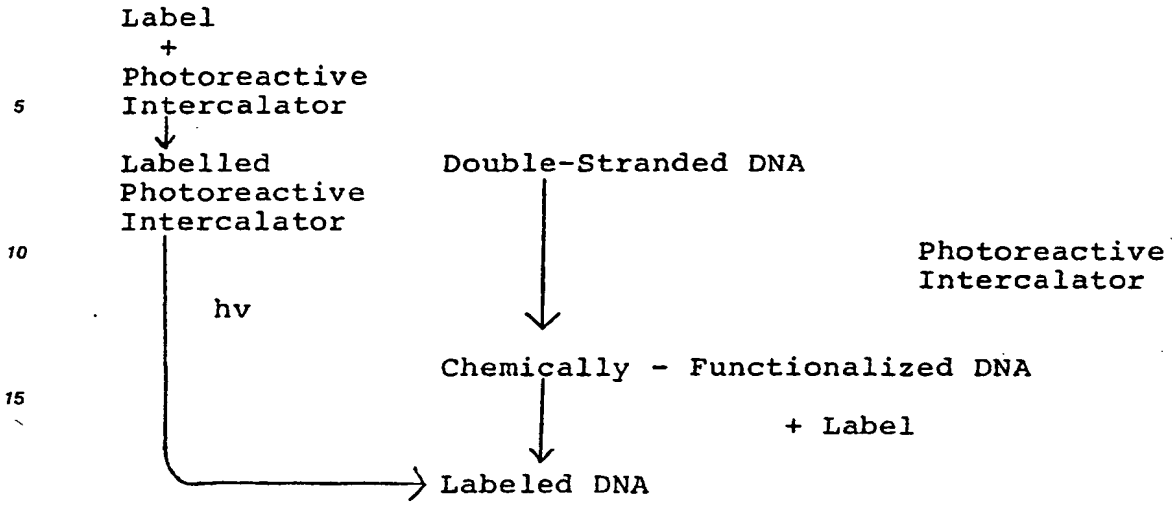
Fig. 2 is a photograph of results of hybridization with labeled genomic DNA for non radioactive detection.

DETAILED DESCRIPTION OF THE INVENTION

The nucleic acid is preferably labeled by means of photochemistry, employing a photoreactive DNA-binding furocoumarin or a phenanthridine compound to link the nucleic acid to a label which can be "read" or assayed in conventional manner, including fluorescence detection. The end product is thus a labeled nucleic acid comprising (a) a nucleic acid component, (b) an intercalator or other DNA-binding ligand photochemically linked to the nucleic acid component, and (c) a label chemically linked to (b).

The photochemical method provides more favorable reaction conditions than the usual chemical coupling method for biochemically sensitive substances. The intercalator and label can first be coupled and then photoreacted with the nucleic acid, or the nucleic acid can first be photoreacted with the intercalator and then coupled to the label.

A general scheme for coupling a nucleic acid, exemplified by double-stranded DNA, to apply a label, is as follows:



Where the hybridizable portion of the nucleic acid is in a double stranded form, such portion is then denatured to yield a hybridizable single stranded portion. Alternatively, where the labeled DNA comprises the hybridizable portion already in single stranded form, such denaturation can be avoided if desired. Alternatively, double stranded DNA can be labeled by the approach of the present invention after hybridization has occurred using a hybridization format which generates double stranded DNA only in the presence of the sequence to be detected.

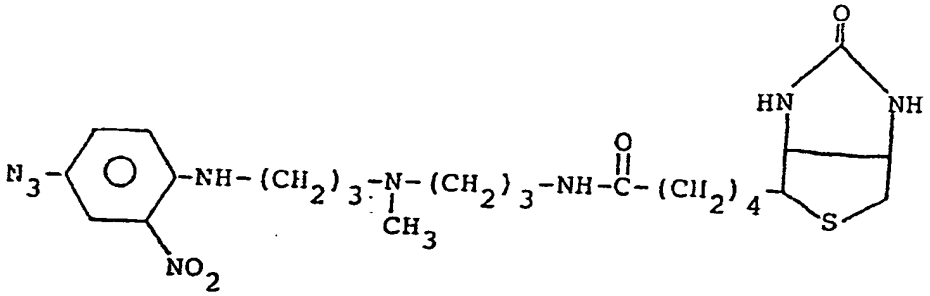
To produce specific and efficient photochemical products, it is desirable that the nucleic acid component and the photoreactive intercalator compound be allowed to react in the dark in a specific manner.

For coupling to DNA, aminomethyl psoralen, aminomethyl angelicin and amino alkyl ethidium or methidium azides are particularly useful compounds. They bind to double-stranded DNA and only the complex produces photoadduct. In the case where labeled double-stranded DNA must be denatured in order to yield a hybridizable single stranded region, conditions are employed so that simultaneous interaction of two strands of DNA with a single photoadduct is prevented. It is necessary that the frequency of modification along a hybridizable single stranded portion of the nucleic acid not be so great as to substantially prevent hybridization, and accordingly there preferably will be not more than one site of modification per 25, more usually 50, and preferably 100, nucleotide bases. Angelicin derivatives are superior to psoralen compounds for monoadduct formation. If a single-stranded DNA nucleic acid is covalently attached to some extra double-stranded DNA, use of phenanthridium and psoralen compounds is desirable since these compounds interact specifically to double-stranded DNA in the dark. The chemistry for the synthesis of the coupled reagents to modify nucleic acids for labeling, described more fully hereinbelow, is similar for all cases.

The nucleic acid component can be single or double stranded DNA or RNA or fragments thereof such as are produced by restriction enzymes or even relatively short oligomers.

The DNA-binding ligands of the present invention used to link the nucleic acid component to the label can be any suitable photoreactive form of known DNA-binding ligands. Particularly preferred DNA-binding ligands are intercalator compounds such as the furocoumarins, e.g., angelicin (isopsoralen) or psoralen or derivatives thereof which photochemically will react with nucleic acids, e.g., 4'-aminomethyl-4,5'-dimethyl angelicin, 4'-aminomethyl-trioxsalen (4'aminomethyl-4,5',8-trimethyl-psoralen), 3-carboxy-5-or -8-amino-or-hydroxy-psoralen, as well as mono-or bis-azido aminoalkyl methidium or ethidium compounds.

Particularly useful photoreactive forms of intercalating agents are the azidointercalators. Their reactive nitrenes are readily generated at long wavelength ultraviolet or visible light and the nitrenes of arylazides prefer insertion reactions over their rearrangement products (see White et al, Methods in Enzymol. , 46, 644 (1977)). Representative intercalating agents include azidoacridine, ethidium monoazide, ethidium diazide, ethidium dimer azide (Mitchell et al, JACS, 104, 4265 (1982)), 4-azido-7-chloroquinoline, and 2-azidofluorene. A specific nucleic acid binding azido compound has been described by Forster et al, Nucleic Acid Res. , 13, (1985), 745. The structure of such compound is as follows:



Other useful photoreactable intercalators are the furocoumarins which form (2+2) cycloadducts with pyrimidine residues. Alkylating agents can also be used such as bis-chloroethylamines and epoxides or aziridines, e.g., aflatoxins, polycyclic hydrocarbon epoxides, mitomycin and norphillin A.

Nonlimiting examples of intercalator compounds for use in the present invention include acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolines.

The label which is linked to the nucleic acid component according to the present invention can be any chemical group or residue having a detectable physical or chemical property, i.e., labeling can be conducted by chemical reaction or physical adsorption. The label will bear a functional chemical group to enable it to be chemically linked to the intercalator compound. Such labeling materials have been well

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